

Potent Activity of Soluble B7-IgG Fusion Proteins in Therapy of Established Tumors and as Vaccine Adjuvant

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ABSTRACT

Fusion proteins consisting of the extracellular region of murine B7.1 or B7.2 and the Fc portion of murine IgG2a (B7-IgG) were evaluated for their ability to promote antitumor responses. Therapeutic administration of soluble B7-IgG in mice with established tumors induced complete regression of the tumor and increased the survival of mice. In three models, MethA, P815, and MB49, mice with 7-day-old established tumors were cured with two to three treatment cycles of B7-IgG, given twice a week. Even in mice with an established B16/F10 tumor (a poorly immunogenic melanoma), therapeutic treatment with B7-IgG alone slowed tumor growth and increased survival significantly. Still stronger antitumor activity was achieved when B7-IgG was used as a vaccine adjuvant mixed with irradiated tumor cells. In 80% of mice with 7-day-old B16 tumors, tumors regressed completely, and mice survived for at least 80 days. In all tumor models, B7.1-IgG and B7.2-IgG had similar antitumor activity. B7-IgG-mediated tumor rejection was dependent on T cells, specifically CD8 cells, as demonstrated by the failure of B7-IgG to induce tumor regression in severe combined immunodeficient or CD8-depleted mice. In addition, mice that were cured of an established tumor were protected against a rechallenge with the same tumor for at least 4 months, suggesting the generation of memory responses. Surprisingly, the antitumor activity of B7-IgG was independent of IFN- γ , as demonstrated by tumor rejection in IFN- γ knockout mice. Our findings demonstrate the potent capacity of B7-IgG to generate or enhance antitumor immune responses and suggest the clinical value of B7-IgG.

INTRODUCTION

Optimal activation of naïve T cells requires signaling through the T-cell receptor as well as through other costimulatory pathways. Naïve T cells stimulated only through the T-cell receptor become nonresponsive, anergic, or die. One such costimulatory signal can be provided by the interaction of B7.1 or B7.2 on APCs² with CD28 on T cells (1-4). However, binding of B7.1 or B7.2 to another receptor on activated T cells, CTLA-4, has been shown to down-regulate immune responses. CTLA-4, a homologue of CD28, binds B7.1 and B7.2 with higher affinity than CD28 and is only expressed on activated T cells. It has been proposed that binding of B7 to CTLA-4 counterbalances CD28-mediated stimulation (1, 2, 5).

Established tumors in mice can be cured by immune-mediated mechanisms (6-8). However, it is also clear that tumors can escape a developing immune response by several mechanisms (7, 9-13). Ineffective activation of T cells due to the absence of appropriate costimulation provides one explanation for inadequate immune responses to growing tumors. It has been proposed that tumor-associated antigens are presented to T cells by nonprofessional APCs lacking costimulatory signals. This suboptimal stimulation leads to

anergy or tolerance of tumor-specific T cells (14-17). Several investigators have had some success in enhancing tumor-specific T-cell activation and/or preventing anergy or tolerance by expressing costimulatory molecules on the surface of tumor cells. Vaccination of mice with tumor cells transfected with B7.1 or B7.2 cDNA protected mice against tumor challenge (18) and induced regression of established tumors in some therapeutic models (17, 19-25). Similarly, direct injection of B7-expressing DNA vectors into tumor-bearing mice has enhanced immune responses and promoted tumor rejection (26). Blocking the interaction of B7 with CTLA-4, thereby preventing negative signals triggered by CTLA-4, has also been used as an approach to enhance antitumor activity (27, 28).

In what may be a more clinically applicable approach to provide B7/CD28-mediated costimulation, we have developed soluble B7-IgG fusion proteins and tested their efficacy in several therapeutic tumor models and protocols. *In vitro* studies with these fusion proteins have demonstrated their costimulatory activity in enhancing proliferation and cytokine production from naïve T cells (29).³ *In vivo* studies using B7.2-IgG as a vaccine adjuvant have demonstrated its capacity to enhance the generation of proliferative and CTL responses to peptide vaccines (30). In the studies reported here, we show that B7.1-IgG or B7.2-IgG mixed with irradiated tumor cell vaccines or administered alone has potent antitumor activity, leading to the regression and cure of established tumors or significantly increased survival of tumor-bearing mice.

MATERIALS AND METHODS

Mice

Female 6-8-week-old BALB/c, C57BL/6, and DBA/2 mice were purchased from either Taconic (Germantown, NY) or The Jackson Laboratory (Bar Harbor, ME). The 6-8-week-old BALB/c-IFN- γ knockout mice were purchased from The Jackson Laboratory. Mice were housed under pathogen-free conditions at Genetics Institute (Andover, MA).

Monoclonal Abs

Rat antimouse CD4 and CD8 monoclonal Abs (GK1.5 and 53-6.72; American Type Culture Collection, Manassas, VA) were produced and purified by standard techniques at Genetics Institute.

B7-IgG Fusion Proteins

Expression plasmids encoding murine B7.1 or B7.2 signal and extracellular domains fused to the Fc region of murine IgG2a were constructed as follows: cDNAs encoding the signal and extracellular domains of murine B7.1 and B7.2 were generated by PCR amplification from cloned cDNA (31, 32). For B7.1, the cDNA extends from the initiation Met in the signal sequence through Asp²⁴³ of the total protein sequence [i.e., Met-Ala-Cys-Asp-Cys... Glu-Asp-Pro-Pro-Asp (31)]. For B7.2, the cDNA extends from the initiation Met in the signal sequence through Pro²³⁹ of the total protein sequence [i.e., Met-Asp-Pro-Arg-Cys... Glu-Phe-Pro-Ser-Pro (32)]. The B7 sequences were joined to a genomic DNA segment encoding the hinge-CH2-CH3 domains for a murine IgG2a Ab (the protein sequence was identical to that of GenBank accession

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² The abbreviations used are: APC, antigen-presenting cell; i.f.p., intra-footpad; i.d., intradermal; FcR, Fc receptor; Ab, antibody; SCID, severe combined immunodeficient; CHO, Chinese hamster ovary; FACS, fluorescence-activated cell-sorting.

³ Unpublished results.

number J00470) such that the IgG2a sequence begins with Glu-Pro-Arg-Gly and ends with Ser-Arg-Thr-Pro. The Cys residues within the Ab hinge region were retained such that the two B7-hinge-CH2-CH3 chains were covalently linked. We also generated fusion proteins (designated B7.1-IgG2mut or B7.2-IgG2mut) in which the IgG2a regions were mutated to ablate binding to Fc- γ RI and complement C1q. The following amino acid residues in the CH2 domain were replaced by Ala: (a) Leu²³⁵; (b) Glu³¹⁸; (c) Lys³²⁰; and (d) Lys³²² (33).

For production of B7-IgG proteins, the reconstructed sequences were inserted in the pHTOP expression vector (34). The recombinant plasmids were transfected into the CHO cell line and amplified by standard techniques (34). CHO cells expressing B7-IgG were grown in DME/F12 (Life Technologies, Inc.) supplemented with 10% FCS, 0.02 μ M methotrexate (34), and 1 mg/ml G418 (Geneticin; Life Technologies, Inc.). At confluence, growth media were discarded, the cells were washed with PBS, and serum-free medium was added. Culture supernatants were collected at 24 h, clarified by sequential passage through 5.0 and 0.22 μ m filters, and concentrated using a 30-kDa tangential flow cartridge filter. The concentrate was loaded onto a protein A-Sepharose Fast Flow column (Pharmacia Biotech), washed with PBS, and eluted with 20 mM citrate (pH 3.0). Elution fractions containing the fusion protein were neutralized with 1 M Tris (pH 8.0; Sigma, St. Louis, MO), and the material was formulated in PBS (pH 7.2) by buffer exchange using a stirred cell with YM30 membrane (Amicon, Beverly, MA). Protein was depyrogenated by chromatography on Poros PI (PerSeptive Biosystems, Framingham, MA). Protein concentration was calculated using an absorbance at 280 nm and a theoretical extinction coefficient of 1.33 cm/mg-mL. More than 99% of the protein was in the dimeric, nonaggregated form, as determined by a TSK 3000 SWXL column [TosoHaas USA, Montgomeryville, PA; PBS (pH 6.8) running buffer]. Endotoxin levels were less than 0.25 endotoxin unit/mg as determined by gel clot assay (Cape Cod Associates).

Tumor Models

All tumor cell lines were cultured in DMEM supplemented with 10% FCS (Sigma) without antibiotics. The following tumor cell lines were used: (a) MethA sarcoma (35); (b) B16/F10 melanoma (36); and (c) MB49 bladder carcinoma (37); P815 and P815-B7.1 were generously provided by T. Gajewski (University of Chicago, Chicago, IL; Refs. 38 and 39). Expression of the transfected mB7.1 in P815-B7.1 was verified by FACS analysis, and the mean fluorescence intensity of 100% of cells was 2.5 log above the unstained control. Solid tumors were established by i.d. or s.c. injection of tumor cells in the flank of the appropriate mouse strain. Five $\times 10^4$ P815 cells were injected into DBA/2 mice; 5×10^5 MethA cells were injected into BALB/c mice; and 2×10^5 B16/F10 cells or 1×10^5 MB49 cells were injected into C57BL/6 mice. Tumor-bearing mice either died within 20–35 days after tumor inoculation (spontaneously metastasizing P815 and B16/F10 tumors) or were sacrificed when the tumors reached a size of approximately 360–400 mm². For purposes of graphic representation of tumor growth, animals that died or had to be sacrificed were assigned a tumor size of 400 mm².

Vaccination Protocols

Prophylactic Protocol. Mice were immunized on day 0 with 1×10^7 irradiated tumor cells in PBS alone or mixed with 75–100 μ g of murine B7.1-IgG, murine B7.2-IgG, or murine IgG. Injections were administered i.f.p. in both hind legs. Mice were also treated with B7-IgG or murine IgG alone on day 5 and challenged on day 7 by i.d. injection in the right flank with live tumor cells (cell number as described above, in 50 μ l).

Therapeutic Protocol. A primary tumor was established by i.d. injection as described above. On day 7–9, when tumors were palpable, mice were vaccinated i.f.p. with 5×10^6 irradiated tumor cells mixed with 25–100 μ g of B7.1-IgG, B7.2-IgG, vehicle (PBS), or irrelevant isotype-matched Ab. Additional B7-IgG, IgG, or vehicle alone was injected i.f.p. 3 days later. This vaccination regimen was repeated weekly for 2–6 weeks.

Therapy with B7-IgG alone was used to treat mice bearing 7-day-old tumors with 25–100 μ g of B7.1-IgG or B7.2-IgG. Injections were i.f.p. twice a week for 2–3 weeks.

Cell Depletions

CD4 or CD8 T cells were depleted by i.p. injections of 100–150 μ g of monoclonal Ab GK1.5 or 53-6.72. Ab treatment was started on day 6, 1 day before initiation of B7-IgG therapy, and continued on days 7, 8, 10, 14, 17, and 21 after tumor inoculation. CD4 or CD8 T-cell depletion was verified by FACS analysis from peripheral blood lymphocytes.

Statistical Analysis

Survival curves were analyzed by the Kaplan-Meier method using the statistical analysis software JMP (version 3.1 for Macintosh; SAS Institute Inc., Cary, NC) according to the manufacturer's specifications.

RESULTS

B7.1-IgG or B7.2-IgG Enhances the Protective Efficacy of an Irradiated Tumor Cell Vaccine. Preliminary studies established that the fusion proteins were effective for costimulation *in vitro* if they were plate-bound, and their activity could be blocked by soluble anti-B7.1 or B7.2 Ab, but not by soluble anti-CTLA-4 Ab (data not shown). These data suggest that, *in vitro*, immobilized B7-IgG provides a costimulatory signal by cross-linking CD28.

To assess *in vivo* function, we first evaluated the activity of B7-IgG fusion proteins in prophylactic tumor vaccine models. Naïve mice were vaccinated with irradiated P815 tumor cells alone or with irradiated cells mixed with B7.1-IgG or B7.2-IgG as described in "Materials and Methods." If included, B7-IgG alone was given again on day 5. Mice were challenged on day 7 with live P815 cells. By day 10, all mice that had not been vaccinated or had been vaccinated with irradiated cells alone developed solid tumors (Table 1). In contrast, 60–70% of mice vaccinated with a combination of irradiated tumor cells and B7.1-IgG or B7.2-IgG were protected against the tumor challenge as assessed by the absence of palpable tumors at day 21. Similar results were obtained in the MethA and B16/F10 tumor models (data not shown).

Our *in vitro* data suggested that aggregation or cross-linking of the B7-IgG proteins was required for costimulation. Therefore, we evaluated the role of the IgG domain and FcR binding in the *in vivo* function of the fusion proteins. Mice were vaccinated with irradiated P815 tumor cells mixed with fusion proteins mutated in the CH2 FcR-binding region (B7.1-IgGmut and B7.2-IgGmut). These mutations are reported to ablate binding to Fc- γ RI receptors and complement (33). In the P815 prophylactic model, the mutated molecules were less effective than the wild-type fusion proteins (Table 1), although both forms exhibited similar costimulatory activity *in vitro* when plate-bound or cross-linked by an antimurine IgG Ab (data not shown). These findings suggest that *in vivo*, in prophylactic models,

Table 1 Protection against tumor challenge after prophylactic vaccination with irradiated tumor cells mixed with B7-IgGs

DBA/2 mice were immunized as described in "Materials and Methods" with irradiated tumor cells mixed with B7-IgG or treated with B7-IgG alone. In all experiments, mice were challenged with 2×10^5 live P815 tumor cells on day 7. Protection against the challenge was determined by the absence of a palpable tumor after 21 days. Protection is shown as the mean percentage (\pm SD) of tumor-free mice from one to five independent experiments.

| Immunization | % Mean protection (\pm SD) | No. of experiments |
|------------------------------------|----------------------------------|-----------------------|
| None | 8 (11) | 5 |
| Irradiated P815 | 2 (4) | 5 |
| Irradiated P815 + B7.1-IgG | 65 (18) | 4 |
| Irradiated P815 + B7.2-IgG | 67 (22) | 5 |
| B7.1-IgG alone | 13 (0) | 2 |
| B7.2-IgG alone | 9 (11) | 4 |
| Irradiated P815-B7.1 transfectant | 23 (4) | 2 |
| Irradiated P815 + B7.1-IgG mutated | 28 (4) | 2 |
| Irradiated P815 + B7.2-IgG mutated | 20 | 1 |

Fc binding of the B7-IgG molecules is important for costimulating unprimed T cells.

Many groups have reported that vaccines of tumor cells expressing membrane-bound B7.1 or B7.2 generate protective antitumor immunity (17-25). We compared the vaccine efficacy of irradiated P815 tumor cells that had been transfected with and expressed high levels of B7.1 (P815-B7.1) with irradiated wild-type P815 cells mixed with B7.1-IgG or B7.2-IgG. Vaccination with irradiated P815-B7.1 cells protected 23% of mice. In contrast, vaccination with irradiated wild-type P815 cells mixed with soluble B7.1-IgG or B7.2-IgG protected 65% and 67% of mice, respectively (Table 1). Similar results were obtained when comparing B16/F10-B7.1 transfectants with B16/F10

cells mixed with the fusion proteins (data not shown). These observations demonstrate the efficacy of soluble B7.1-IgG and B7.2-IgG as vaccine adjuvant and indicate that they may be more potent than tumor cell transfectants expressing high levels of membrane-bound B7.1.

Therapeutic Vaccination with Irradiated P815 Tumor Cells Mixed with B7.1-IgG or B7.2-IgG Cures Mice of Established P815 Tumor. To test the adjuvant activity of B7-IgG in a therapeutic tumor vaccine model, DBA/2 mice with 7-day-old s.c. P815 tumors were injected i.f.p. with irradiated P815 tumor cells alone or mixed with B7.1-IgG or B7.2-IgG. A second dose of B7-IgG alone was administered i.f.p. 3-5 days later. This treatment was repeated weekly for 3

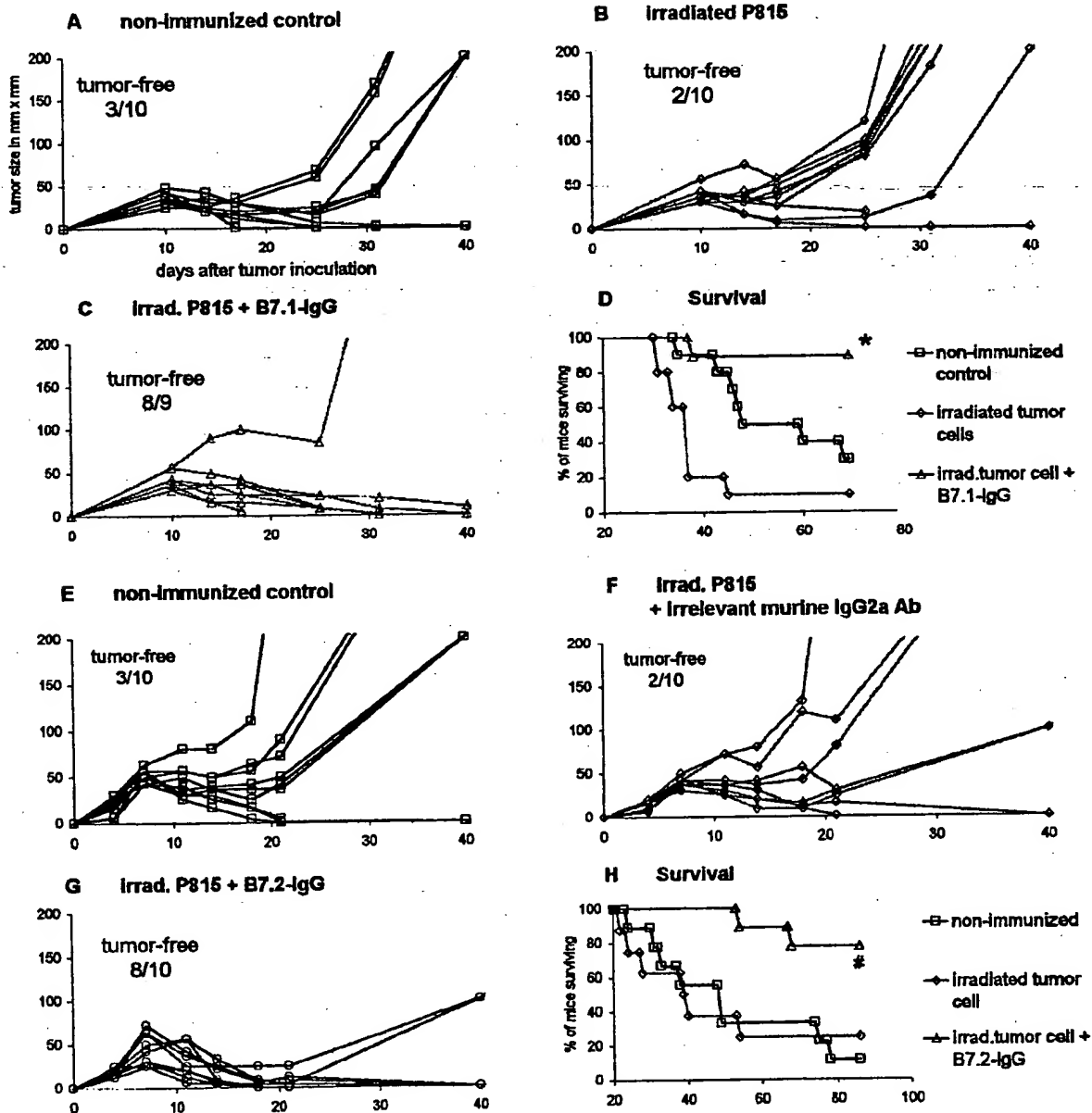


Fig. 1. Vaccination of P815 tumor-bearing mice with irradiated P815 tumor cells mixed with B7.1-IgG or B7.2-IgG induces tumor regression and prolongs survival. P815 tumors were established on day 0 by i.d. injection of 5×10^4 P815 cells. Mice were vaccinated on day 7 by i.f.p. injection with either PBS (A and E), irradiated P815 tumor cells (B), tumor cells mixed with irrelevant mouse IgG2a Ab (F), or irradiated P815 cells mixed with B7.1-IgG (C and G). PBS, irrelevant Ab, or the same B7-IgG as used initially was readministered 3 days later. The vaccination cycle was repeated for 3 weeks. Tumor growth was monitored, and the number of tumor-free mice after 40 days is indicated. In A, B, C, E, F, and G, each line represents the tumor growth of an individual mouse. Mice that were euthanized or died of metastatic disease were assigned a tumor size of 400 mm². D and H show the percentage of surviving animals/group as a function of time. Survival of mice treated with B7.1-IgG or B7.2-IgG was significantly increased compared with the controls (*, $P < 0.017$; #, $P < 0.0027$, respectively, by log-rank test). The data are representative of four independent experiments.

weeks. Beginning about 1 week after the first immunization, reduced tumor growth and tumor regression were observed in mice treated with tumor cells mixed with either of the B7-IgGs (Fig. 1, C and G). Tumor growth was not reduced in most mice treated with irradiated tumor cells alone or with cells mixed with an irrelevant mouse IgG2a Ab (Fig. 1, B and F). In five independent experiments, primary tumors disappeared in 60–90% of mice treated for three cycles with irradiated P815 tumor cells mixed with either B7.1-IgG or B7.2-IgG, compared with 10–30% in the control groups. Regression of the primary tumor also correlated with statistically significant increases in survival (Fig. 1, D and H).

B7-IgG as Therapeutic Tumor Vaccine Adjuvant in Different Tumor Models. To demonstrate that the results with B7-IgG were not unique to the P815 tumor or the DBA/2 mouse strain, we tested the efficacy of B7-IgG as an adjuvant in three additional tumor models in two other mouse strains. BALB/c mice bearing 7-day-old s.c. MethA sarcomas were treated with PBS or irradiated MethA cells alone or mixed with either B7.1-IgG or B7.2-IgG. Two vaccinations with irradiated MethA cells mixed with B7-IgG led to complete tumor regression in 90–100% of mice (Fig. 2, C and D). Mice were tumor free 60–90 days later. In groups left untreated, treated with irradiated MethA cells alone (Fig. 2, A and B), or treated with irradiated cells mixed with an irrelevant mouse IgG2a (two independent studies; data not shown) tumors regressed in only 10% of mice. We obtained comparable results with MB49 tumor cells in C57BL/6 mice (Fig. 2, E–H).

In the poorly immunogenic melanoma model, B16/F10 (in C57BL/6 mice), therapeutic vaccination with irradiated tumor cells and B7-IgG proteins significantly reduced tumor growth and increased long-term survival (Fig. 2, I–L). By day 35, all mice left untreated or vaccinated with irradiated cells alone had succumbed to the tumor or were euthanized with large tumor masses (Fig. 2, I, J, and L). In contrast, 60% of mice immunized with the B7.1-IgG vaccine (data not shown) and 90% of mice immunized with the B7.2-IgG vaccine survived for >80 days (Fig. 2, K and L).

Therapeutic Administration of B7-IgG Alone Induces Antitumor Responses. The potent adjuvant activity of B7.1-IgG and B7.2-IgG in the therapeutic vaccine models prompted us to test their efficacy as therapeutic agents alone. In all four therapeutic tumor models, treatment with B7.1-IgG or B7.2-IgG alone reduced tumor growth and increased survival (Fig. 3). In three of the models, the efficacy of treatment with B7-IgG alone was similar to that of vaccination with irradiated cells mixed with B7-IgG (Fig. 3, A–C). In the B16/F10 model, treatment with B7-IgG alone slowed tumor growth and significantly increased survival time. However, tumor cure in 80% of B16/F10 tumor-bearing mice could only be achieved by vaccination with irradiated tumor cells and B7.1-IgG or B7.2-IgG as adjuvant (Fig. 3D). The B16/F10 tumor was also the only model in which B7.2-IgG was slightly more effective than B7.1-IgG. In all other models, B7.1-IgG and B7.2-IgG had similar activity.

In contrast to the prophylactic vaccine models, where efficacy was reduced for fusion proteins mutated in the Fc binding domain, therapeutic vaccination with the mutated forms of B7-IgG was as efficacious as treatment with the wild-type forms (data not shown). These data suggest that the activity of B7-IgG in a therapeutic setting does not require FcR binding and demonstrate the potency of B7-IgG as antitumor agent.

B7-IgG-mediated Tumor Cure Is CD8 but not CD4 T-Cell Dependent and Generates Lasting Protective Immunity. To determine whether the antitumor response mediated by B7-IgG is dependent of the adaptive immune response, we evaluated B7-IgG therapy in SCID mice. BALB/c-SCID mice bearing MethA tumors were treated with B7.2-IgG alone or mixed with irradiated MethA cells.

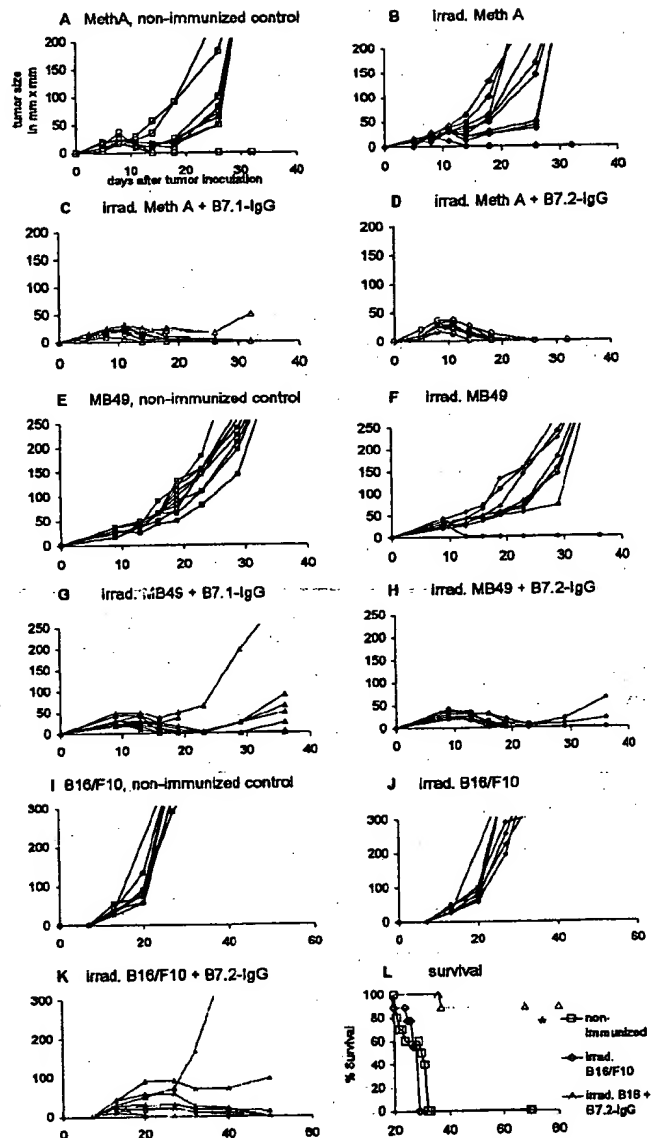


Fig. 2. Vaccination with B7-IgG as an adjuvant is effective in three other therapeutic tumor models. Solid tumors were established on day 0 as described in "Materials and Methods." A total of 8–10 mice/group were vaccinated, starting on day 7, by i.f.p. injection with either irradiated tumor cells (B, F, and J) or cells mixed with 25 (C and D), 50 (G and H), or 100 μ g (K) of B7.1-IgG or B7.2-IgG, respectively. PBS, B7.1-IgG, or B7.2-IgG, as used in the initial injection, was given again 3–4 days later. One group was treated with PBS alone (A and E; data not shown for B16/F10; IgG2a isotype controls were included in two experiments). Tumor growth was monitored for 35–60 days. Mice with MethA or MB49 tumors were euthanized once the tumor reached a size of about 360 mm³. B16/F10-bearing mice either died from metastatic disease or were euthanized when their tumors reached about 360 mm³. In A–K, each line represents the tumor growth of an individual mouse. The percentage of surviving animals/group is shown in L (*, $P < 0.0001$ by log-rank test). Experiments were repeated two to five times with comparable results.

Neither treatment had an effect on tumor growth (Fig. 4), proving the dependence of B7-IgG-mediated tumor responses on T or B cells. To further define the T-cell subsets important for the antitumor activity of B7-IgG, we treated MethA tumor-bearing mice after depleting CD8 or CD4 T cells. Depletion of CD8 or CD4 T cells by Ab injection was started 6 days after tumor inoculation, i.e., 1 day before initiation of B7-IgG therapy. Successful depletion was verified by FACS analysis of peripheral blood lymphocytes on day 28: CD4 cells were undetectable; and CD8 cells were <1.5% of peripheral blood lymphocytes. In

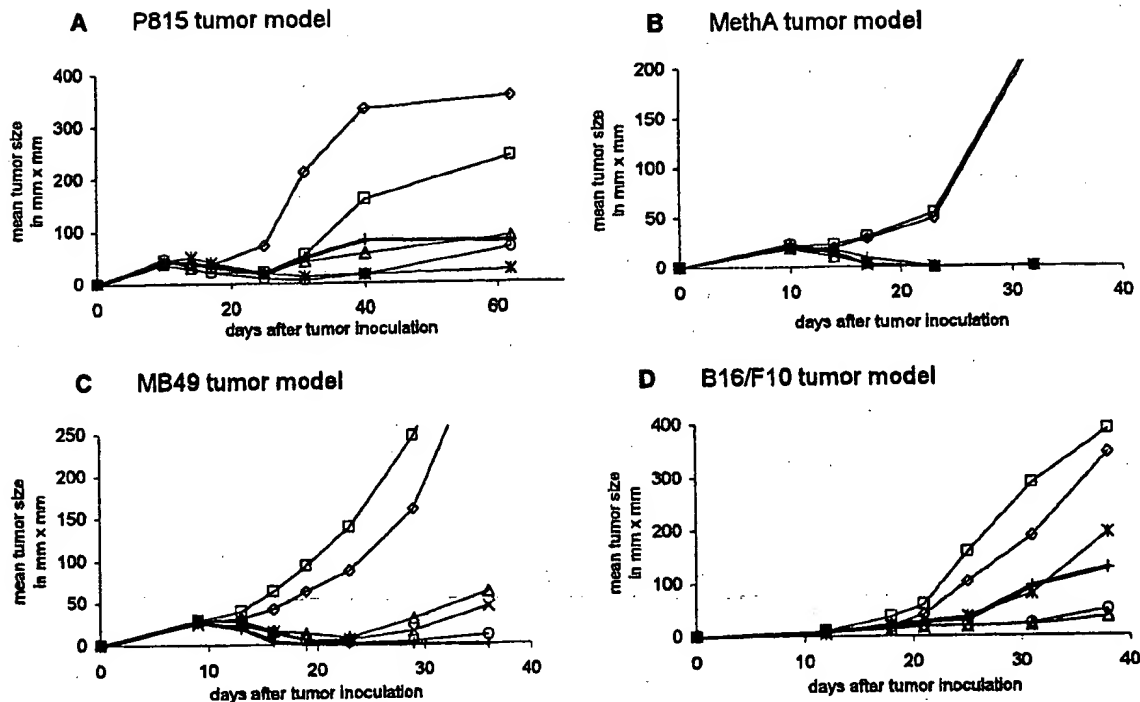


Fig. 3. Therapeutic administration of B7-IgG alone has antitumor activity. Mice were inoculated on day 0 with live P815 (A), MethA (B), MB49 (C), or B16/F10 (D) tumor cells as described in "Materials and Methods." Treatment started on days 6–8. Mice were treated with PBS (□), irradiated tumor cells alone (◇), irradiated tumor cells mixed with B7.1-IgG (Δ), or B7.2-IgG (○) or with B7.1-IgG (∗) or B7.2-IgG alone (+). The mean tumor size/group (groups of 7–10 mice) is plotted. Mice were euthanized when tumor size reached 360 mm², and mice that died of metastatic disease were assigned a tumor size of 400 mm².

CD4-depleted mice, the growth of MethA tumors was comparable with that in untreated normal mice (Fig. 5, A and B). Therapy with B7.2-IgG induced complete tumor regression and cure in CD4-depleted mice (Fig. 5C). In contrast, treatment with B7.2-IgG in CD8-

depleted mice slowed tumor growth but did not cure the mice (Fig. 5, D and E). Thus, depletion of CD8 T cells abrogated the antitumor activity of B7.2-IgG.

Further evidence that B7-IgG therapy of established tumors de-

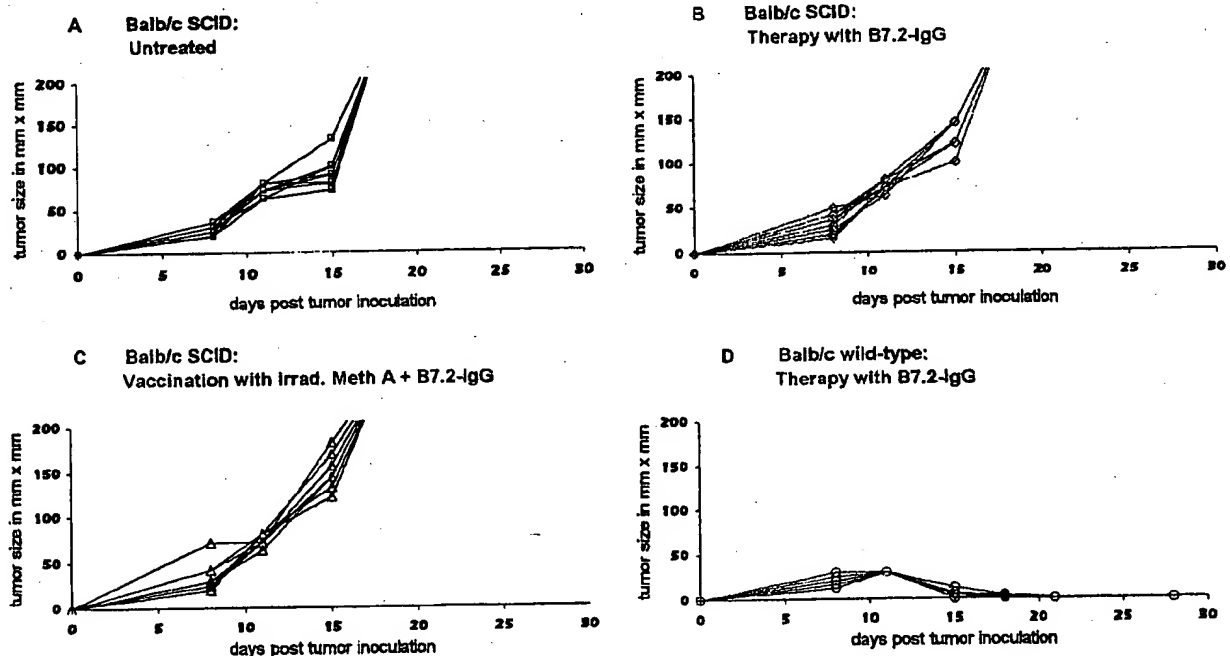


Fig. 4. B7-IgG-mediated antitumor responses depend on T and B cells. Normal or SCID BALB/c mice were injected with 2×10^5 MethA tumor cells. Treatment of established tumors was started 7 days later (except for untreated control mice in A). Mice were treated for 3 weeks with 50 μ g of B7.2-IgG twice a week (B and D) or with 5×10^6 irradiated MethA cells mixed with 50 μ g of B7.2-IgG followed by an additional dose of 50 μ g of B7.2-IgG 3 days later (C).

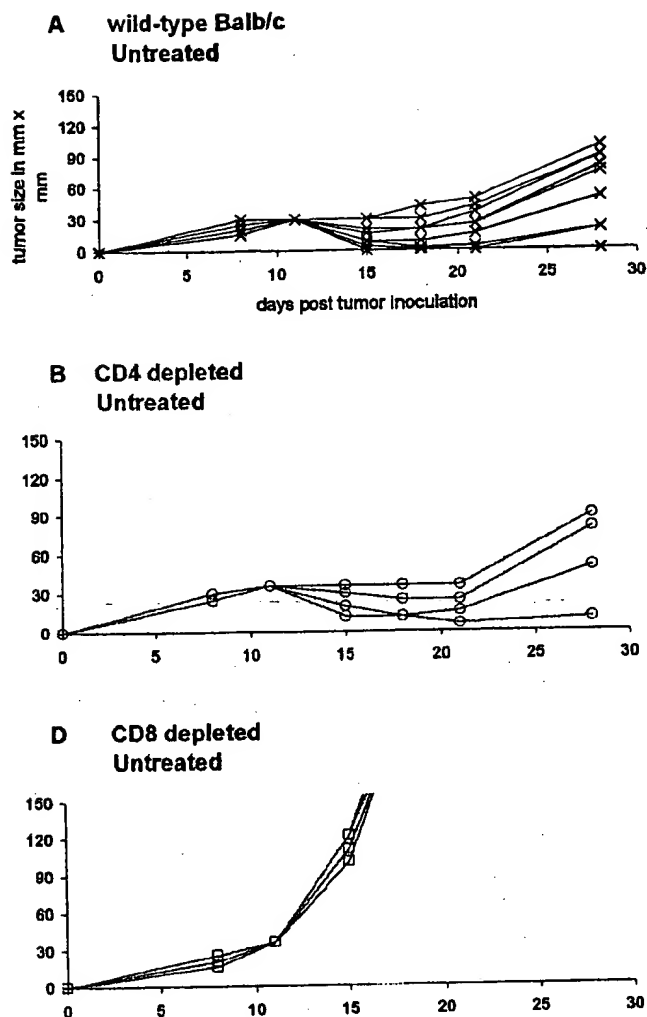


Fig. 5. B7-IgG therapy of established tumors is dependent on CD8 cells but not CD4 T cells. Solid tumors were established in BALB/c mice by injection of 2×10^5 MethA cells. CD4 or CD8 T cells were depleted by Ab treatment beginning on day 6 as described in "Materials and Methods." Therapy with 50 μ g of B7.2-IgG was given on days 7, 10, 14, and 17 in the groups shown in C and E. Groups shown in A, B, and D received no treatment with B7-IgG.

depends on tumor-specific immune responses is provided by rechallenge experiments. Mice that had been cured of established P815 or MethA tumors by therapy with B7.1-IgG or B7.2-IgG were rechallenged with the same tumors on the contralateral flank 60–110 days after the last vaccination. None of the rechallenged mice developed palpable tumors, regardless of whether they had been previously treated with B7.1-IgG or B7.2-IgG alone or with irradiated tumor cells mixed with either B7-IgG (data not shown). In total, these findings indicated that B7-IgG-enhanced antitumor responses were dependent on CD8 T cells and that memory responses were generated in mice cured by B7-IgG therapy.

B7-IgG-mediated Tumor Therapy Is IFN- γ Independent. Because IFN- γ plays an important role in antitumor immune surveillance and antitumor responses (40–42), we asked whether B7-IgG could cure established tumors independent of IFN- γ . Normal BALB/c or BALB/c-IFN- γ knockout mice bearing 7-day-old MethA tumors were treated with B7.2-IgG twice a week for 3 weeks or with irradiated tumor cells mixed with B7.2-IgG once a week for 3 weeks. Tumors grew more rapidly in the untreated IFN- γ -deficient mice compared with wild-type mice (Fig. 6, A and D). However, in both mouse strains, therapeutic treatment with B7.2-IgG alone (Fig. 6, B and E) or with irradiated tumor cells plus B7.2-IgG (Fig. 6, C and F) induced tumor regression and cure by about day 28. These results demonstrated that B7.2-IgG tumor therapy is IFN- γ independent.

DISCUSSION

We report here that fusion proteins linking B7.1 or B7.2 extracellular domains to the Fc region of murine IgG2a function as potent adjuvants for antitumor vaccines. B7.1-IgG or B7.2-IgG mixed with an irradiated tumor cell vaccine protected mice against a tumor challenge. More significantly, irradiated tumor cells mixed with B7.1-IgG or B7.2-IgG as an adjuvant were effective as therapeutic tumor vaccines in three strains of mice and in four tumor models. In all four tumor models, vaccination of tumor-bearing mice with irradiated tumor cells mixed with B7.1-IgG or B7.2-IgG led to regression of solid tumors and cure as measured by survival without detectable primary tumor for an observation period of 70–120 days. The potency of B7-IgG was best demonstrated in the poorly immunogenic B16/F10 melanoma model, where we achieved cure in up to 90% of the mice (Fig. 2). Most importantly, therapeutic treatment of tumor-bearing mice with B7-IgG alone also induced significant tumor regression and increased survival in all models tested. In fact, in three models (P815, MethA, and MB49) the results after treatment with B7-IgG alone were indistinguishable from treatments with irradiated tumor cell vaccines mixed with B7-IgG (Fig. 3). The B16/F10 tumor was the only model in which immunization with irradiated tumor mixed with either B7-IgG had a greater effect than treatment with B7-IgG alone (80% long-term survival *versus* 40% long-term survival, respectively). In this tumor model also, B7.2-IgG had a some-

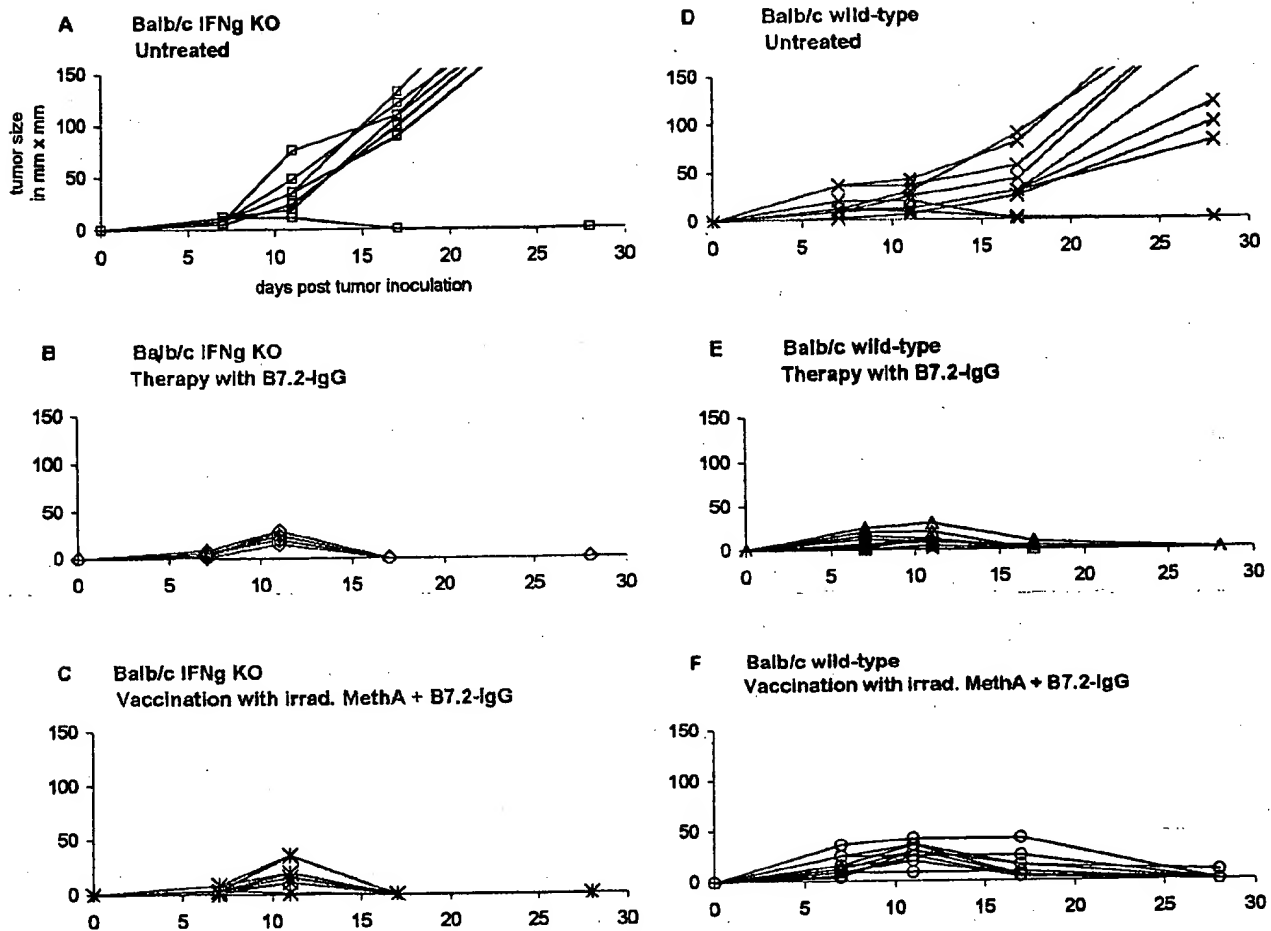


Fig. 6. B7-IgG therapy cures established MethA tumors in the absence of IFN- γ . MethA tumors were established in normal BALB/c mice (D and F) or IFN- γ knockout BALB/c mice (A-C) on day 0. Treatment with B7-IgG was started on day 7, with 50 μ g of B7.2-IgG given twice a week for 3 weeks (B and E). Groups shown in C and F were vaccinated with irradiated MethA tumor cells mixed with 50 μ g of B7.2-IgG on days 7, 14, and 21 and an additional dose of 50 μ g of B7.2-IgG on days 10, 14, and 24. Mice in the groups shown in A and D were left untreated.

what greater effect than B7.1-IgG. In summary, both B7.1-IgG and B7.2-IgG demonstrated strong antitumor activity in several established tumor models as therapeutic agents and as a vaccine adjuvant.

To determine whether the therapeutic antitumor activity of B7-IgG is immune-mediated, we tested B7.2-IgG in tumor-bearing mice lacking T and B cells, depleted of CD4 or CD8 cells, or lacking the capacity to generate IFN- γ . The absence of B7.2-IgG activity in SCID mice established the role of T and/or B cells (Fig. 4). Studies of mice depleted of CD4 or CD8 cells by Ab treatment revealed that CD8 cells are essential for the function of B7-IgG (Fig. 5). The role of CD4 cells may not be as clear. Although B7.2-IgG was active in mice depleted of CD4 cells, suggesting that CD4 cells are not essential for B7-IgG therapy, it is important to indicate that CD4 depletion was initiated 6 days after tumor inoculation. Thus, there may have been time to generate tumor-specific CD4 responses and to provide help in the initiation phase of the CD8 response. Depletion of CD4 T cells at the time of tumor inoculation may reveal a role for this T-cell subset. Nevertheless, our data show that CD8 cells, but not CD4 cells, need to be present during B7-IgG therapy. The rechallenge experiments provide further proof that the activity of B7-IgG is mediated through tumor-specific immune mechanisms and that memory responses are generated. It remains to be determined whether B7-IgG induces a new tumor-specific response or promotes the expansion and enhancement of existing yet ineffectual responses.

Perhaps the most remarkable finding is that the therapeutic effect of B7-IgG is independent of IFN- γ expression by the host. Therapeutic treatment with B7.2-IgG led to tumor cure in antitumor IFN- γ knockout mice (Fig. 6). The capacity of B7.2-IgG to enhance activity independent of IFN- γ is consistent with our observations in other studies that B7-IgG as a vaccine adjuvant enhances but does not shift type 1 and type 2 immune responses (30). This property of B7-IgG distinguishes it from other immune therapeutic approaches such as interleukin 2, IFN- γ , and interleukin 12, where IFN- γ plays a key role (43, 44). We are currently investigating other cytokines that may be responsible for the effects of B7-IgG. Granulocyte macrophage colony-stimulating factor is especially interesting because it has been shown to have potent antitumor activity when given as a vaccine adjuvant or transfected into tumor cells (45, 46). Moreover, cross-linking of CD28 has been shown to induce granulocyte macrophage colony-stimulating factor.

Several hypotheses can be proposed for the mechanism by which the B7-IgG fusion proteins assist the generation of novel antitumor responses or enhance existing antitumor responses. B7.1-IgG and B7.2-IgG can bind to both CD28 and CTLA-4 (as demonstrated by *in vitro* binding studies; data not shown). Therefore, B7-IgG has the potential to enhance costimulatory effects through CD28 as well as to prevent negative signals triggered through CTLA-4. The role and effect of B7-IgG may shift, depending on the condition of the T-cell

response. For naïve T cells, which initially do not express CTLA-4, costimulation through CD28 is likely to be most important. CD28 signaling may be increased by aggregation of B7-IgG, mediated through Fc binding on APCs. This is consistent with our observation that in prophylactic models, B7-IgG requires the Fc binding function (Table 1). In contrast, in therapeutic settings, the stimulation of activated or memory T cells recognizing tumor antigens may require less costimulation through CD28 (47). Thus, in therapeutic settings, aggregation of B7-IgG may be less important, which is consistent with our finding that in this circumstance, wild-type and Fc-mutated forms of B7-IgG are equally effective (data not shown). In addition, in therapeutic settings, soluble B7-IgG may bind with high affinity to CTLA-4 on activated T cells to block its negative signal, thereby enhancing the activity of tumor-specific T cells or preventing their down-regulation. A better characterization of the function of B7-IgG remains the focus of future studies and will help us to understand the differences in various tumor therapy approaches targeting the B7-CD28/CTLA-4 costimulatory pathway.

Previous reports described the successful therapeutic treatment of established murine tumors with Ab blocking CTLA-4 (27). This approach induced the regression of strongly immunogenic tumors but not poorly immunogenic tumors (40), in contrast to our findings that B7.1-IgG and B7.2-IgG were effective as therapeutic agents or vaccine adjuvant even in the poorly immunogenic tumors MB49 and B16/F10. These observations suggest that B7-IgG may work through different mechanisms than anti-CTLA-4 Ab, has greater potency in blocking CTLA-4, or has a dual function in binding to CD28 and CTLA-4. Another approach targeting the costimulation pathway in tumor therapy focuses on transfection or transduction of B7 into tumor cells. However, this approach has only been successful in prophylactic tumor models (17–25). Little success using B7-transfected tumor cells has been achieved in therapeutic tumor models, as corroborated by our findings with the B7.1 transfectants of P815 and B16/F10 (Table 1; data not shown). The relative ineffectiveness of tumor cells transfected with B7 as compared with injection of soluble protein may reflect quantitative differences in the number of available B7 molecules. However, the difference may also be explained by the dual function of B7 molecules. Membrane-bound B7 expressed by APCs can cross-link CD28 and costimulate T cells, but it can also cross-link CTLA-4 on activated T cells and trigger a negative signal. B7 expressed on transfected tumor cells may have comparable functions. In contrast, soluble B7-IgG may block rather than trigger signaling through CTLA-4, sustaining the activation of tumor-specific T cells. Studies to better understand the mechanism of the immune-enhancing effect of B7-IgG are in progress.

Recently, Moro *et al.* (48) reported the therapeutic antitumor efficacy of B7-immunoglobulin fusion proteins. Their approach is comparable with vaccination with B7-transfected tumor cells because they indirectly targeted B7-immunoglobulin to the tumor with Ab recognizing tumor-specific antigens. In contrast to our findings, they did not observe antitumor activity when B7-immunoglobulin was administered in soluble form without the targeting Ab. Without having compared the different fusion proteins, we could speculate that the differences in production and the tagging with biotin may affect the pharmacokinetics of the molecules. In preliminary studies, we have determined long half-lives of 80–90 h in mice for our B7-IgG proteins. It is worth noting that despite the extended systemic exposure with these costimulatory molecules, we have not observed any morbidity or signs of autoimmune diseases in mice kept for more than 4 months after therapy.

In summary, B7-IgG fusion proteins appear to be effective antitumor agents that seem to be safe and can readily be administered in the clinic and manufactured. Their potency in stimulating immune re-

sponses and cure in multiple murine tumor models suggests clinical potential as an adjuvant and therapy for oncology and for other clinical indications.

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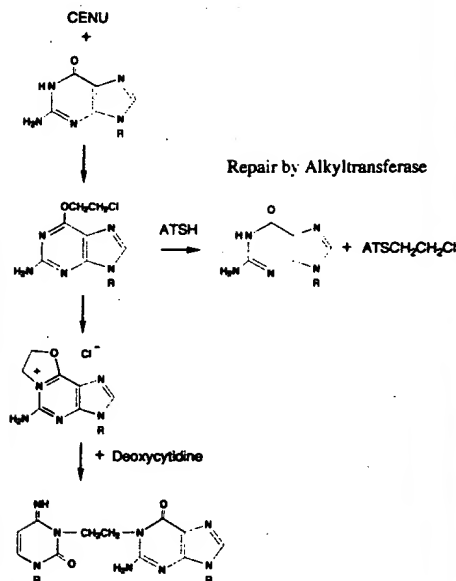
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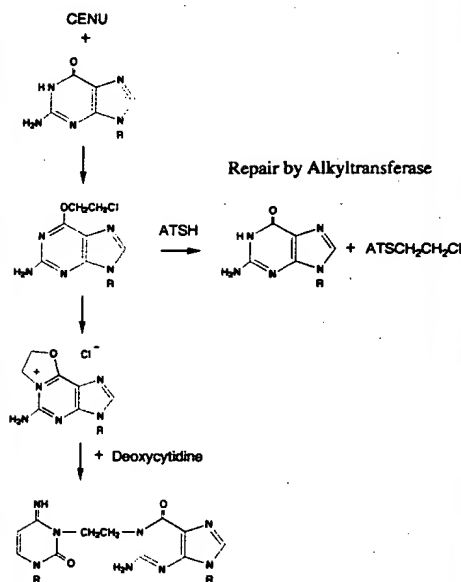


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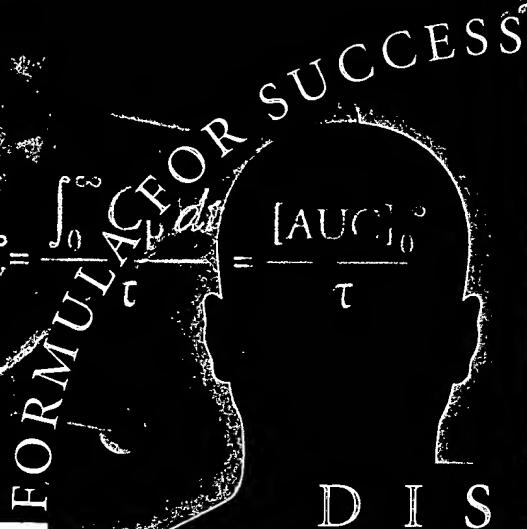
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